Comparison of different live/dead stainings for detection and quantification of adherent microorganisms in the initial oral biofilm

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Abstract: OBJECTIVES: The aim of the present study was to investigate different fluorescence-based, two-color viability assays for visualization and quantification of initial bacterial adherence and to establish reliable alternatives to the ethidium bromide staining procedure. MATERIALS AND METHODS: Bacterial colonization was attained in situ on bovine enamel slabs (n = 6 subjects). Five different live/dead assays were investigated (fluorescein diacetate (FDA)/propidium iodide (PI), Syto 9/PI (BacLight®), FDA/Sytox red, Calcein acetoxymethyl (AM)/Sytox red, and carboxyfluorescein diacetate (CFDA)/Sytox red). After 120 min of oral exposure, analysis was performed with an epifluorescence microscope. Validation was carried out, using the colony-forming units for quantification and the transmission electron microscopy for visualization after staining. RESULTS: The average number of bacteria amounted to $2.9 \pm 0.8 \times 10(4)$ cm(-2). Quantification with Syto 9/PI and Calcein AM/Sytox red yielded an almost equal distribution of cells (Syto 9/PI 45 % viable, 55 % avital; Calcein AM/Sytox red 52 % viable, 48 % avital). The live/dead ratio of CFDA/Sytox red and FDA/Sytox red was 3:2. An aberrant dispersal was recorded with FDA/PI (viable 34 %, avital 66 %). The TEM analysis indicated that all staining procedures affect the structural integrity of the bacterial cells considerably. CONCLUSION: The following live/dead assays are reliable techniques for differentiation of viable and avital adherent bacteria: BacLight, FDA/Sytox red, Calcein AM/Sytox red, and CFDA/Sytox red. These fluorescence-based techniques are applicable alternatives to toxic and instable conventional assays, such as the staining procedure based on ethidium bromide. CLINICAL RELEVANCE: Differentiation of viable and avital adherent bacteria offers the possibility for reliable evaluation of different mouth rinses, oral medication, and disinfections.

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Clinical Oral Investigations

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Abstract

Objectives: The aim of the present study was to investigate different fluorescence-based, two-colour viability assays for visualization and quantification of initial bacterial adherence and to establish reliable alternatives to the ethidium bromide staining procedure.

Materials and Methods: Bacterial colonization was attained *in situ* on bovine enamel slabs (n = 6 subjects). Five different live/dead assays were investigated (FDA/ PI, Syto 9/ PI [BacLight®], FDA/ Sytox red, Calcein AM/ Sytox red, CFDA/ Sytox red). After 120 min of oral exposure, analysis was performed with an epifluorescence microscope. Validation was carried out, using the colony forming units for quantification- and the transmission electron microscopy for visualisation after staining.

Results: The average number of bacteria amounted to $2.9 \pm 0.8 \times 10^4$ cm^{-2}. Quantification with Syto 9/ PI and Calcein AM/ Sytox red yielded an almost equal distribution of cells (Syto 9/ PI: 45% viable, 55 % avital; Calcein AM/ Sytox red: 52% viable, 48% avital). The live/dead ratio of CFDA/ Sytox red and FDA/ Sytox red was 3:2. An aberrant dispersal was recorded with FDA/PI (viable: 34%, avital: 66%). The TEM analysis indicated that all staining procedures affect the structural integrity of the bacterial cells considerably.

Conclusion: The following live/dead assays are reliable techniques for differentiation of viable and avital adherent bacteria: BacLight, FDA/ Sytox red, Calcein AM/ Sytox red and CFDA/ Sytox red. These fluorescence-based techniques are applicable alternatives to toxic and instable conventional assays, such as the staining procedure based on ethidium bromide.

Clinical Relevance: Differentiation of viable and avital adherent bacteria offers the possibility for reliable evaluation of different mouth rinses, oral medication and disinfections.

Keywords live/dead staining, viability, adherent bacteria, initial biofilm, CFU, TEM

Introduction

Prior to bacterial biofilm formation a proteinaceous layer, known as the acquired pellicle, covers all oral tissues within seconds. Consisting of different salivary compounds, initial bacterial colonizers and different enzymes, the pellicle provides the base for biofilm formation in the oral cavity [1, 2]. It has been pointed out that the initial bioadhesion constitutes a focal point in basic science questions as well as in research areas of protein adsorption, mineralisation/demineralisation, bacterial adherence and antimicrobial activity [3- 5]. Therefore the investigation of the characteristics of the acquired pellicle and the initial bacterial adherence is of great relevance and reveals options how to influence and ultimately reduce biofilm formation on oral surfaces [6, 7].

Besides the common bacterial culturing techniques for the detection of single bacteria and bacterial aggregates, studies have been performed to visualize the adherent microorganisms with fluorogenic dyes. Disadvantages of the viable plate count and culture-based techniques are the selection for certain bacteria. Moreover the preparation for the culturing techniques to detect bacteria in the biofilm requires desorption procedures and dispersion onto the agar plates, which may influence the results considerably [1]. Over 50% of the oral bacterial flora is not culturable [8]. Therefore, simple methods for direct visualization and quantification of bacteria in their physiological adherent state are more convenient. This is possible with fluorescence staining techniques followed by fluorescence microscopic analysis [9, 10]. While fluorescence microscopic methods such as FISH or DAPI offer the opportunity to mark selected bacterial species or to stain all cells, there are furthermore techniques, which allow the differentiation of viable and dead bacteria [11-14]. This differentiation, based either on membrane permeability or metabolic activity, offers the possibility to compare the percentaged live/dead distribution of the bacteria before and after treatment with different rinses or other adverse environmental conditions [15-17].

One of the first dyes used for the detection of viable cells was fluorescein diacetate (FDA), the uncoloured precursor of fluorescein. This dye develops its fluorescence after intracellular metabolism and accumulates in the cell, emitting green light after excitation with 490 nm [18-21].

Requiring the enzymatic metabolism by esterase, only viable cells show fluorescence. In comparison, carboxyfluorescein diacetate (CFDA) shows a longer intracellular accumulation, due to its negative charge [12, 19, 22]. Calcein AM, another fluorescing dye for vital cells, is readily cleaved by intracellular esterases into the fluorescent calcein. Once turned into this fluorescing state, it distributes throughout the whole cell, nuclei and mitochondria included

with green colour (emission peak 520 nm) [23]. Other live/dead stains such as SYTO 17 or Syto 9 green are based on the intercalation into DNA or RNA [24]. These dyes have the ability to penetrate intact membranes and contrasting cell nuclei and chromatin for live cell fluorescence microscopy [24].

One of the first staining assays for the detection of dead bacteria was ethidium bromide (Etbr). After the transport of the huge ethidium bromide molecules via passive diffusion processes into dead cells with permeable membranes, the molecules intercalate into DNA and show an enhancement of fluorescence [25-27].

However, safety data sheets of several manufacturers and the positive Ames test suggest the mutagenicity of ethidium bromide [28]. Alternatives are offered by membrane-impermeable nucleic acid SYTOX® dead cell stains. These stains are non-fluorescent in aqueous solutions and exhibit up to a 100-fold increase in fluorescence upon nucleic acid binding. Sytox red, for example, is a high-affinity nucleic acid stain that easily penetrates cells with compromised membranes, while uncompromised cell membranes are excluded [29].

Propidium iodide (PI) also penetrates damaged membranes of dead cells only. Loss of the permeability barrier of stained cells represents irreparable damage and thus cell death.

The wide range of fluorescence-based dyes, differing in their target area and way of cell penetration, makes it difficult to obtain the overview and apply adequate materials. Besides the differences in excitation and emission spectrum the dyes also seem to lead to differently percentaged distributions of viable and dead bacteria due to the smooth transition of the viability states [12, 30, 31].

Hence, the aim of the present *in situ* study was the investigation of different live/dead staining techniques for adequate visualisation and quantification of microorganisms during the first phase of oral bioadhesion to enamel and to establish reliable alternatives to the ethidium bromide staining procedure.

Material and methods

Subjects and specimens

Six healthy volunteers participated in this study. A prior anamnesis and visual oral examination ensured that the subjects were free of gingivitis, caries and general diseases. The study was approved by the Ethics Committee of the University of Freiburg (proposals 222/08, 239/08). Cylindrical enamel slabs (diameter 5 mm, 19.63 mm^2 surface area, height 1.5 mm) were prepared from labial surfaces of bovine incisors of BSE-negative cattle. The enamel surfaces of all samples were polished by wet grinding with abrasive paper up to 4000 grit. One day before the oral exposure, cleaning and disinfection of the slabs was carried out by ultrasonication in different solutions: after treatment in NaOCl (3%) for 2 min to remove the superficial smear layer, 3- and 10-min min ultrasonications in ethanol (70%) and aqua bidest., respectively, were performed. To ensure the formation of a hydration layer, the enamel slabs were stored in aqua bidest. for at least 24 h. The slabs were fixed in the 2^{nd} premolar and 1^{st} and 2^{nd} molar regions on the buccal sites of individual upper jaw splints with a polysiloxane impression material (Aquasil Ultra, Dentsply DeTray GmbH, Konstanz, Germany). The enamel surface was embedded on the splint with the margin completely covered by the impression material. Covering the margin was necessary to ensure the exposure of the surface solely. Altogether, a total of five different live/dead assays and CFU (colony forming units) were investigated, using two enamel slabs for each method. For reproducibility of the slab location on the splint, two throughputs were performed by the subjects, respectively. The specimens were exposed in the oral cavity for 120 min. Afterwards, the specimens were rinsed for 10 s with sterile 0.9% saline solution. Subsequently, adherent bacteria were visualized and quantified by the different staining techniques and the CFU.

Live/Dead Staining

Ten different live/dead assays were evaluated in a preliminary test to detect and to quantify oral bacteria in the initial biofilm (tab. 1). For main experiments, the following five staining combinations were selected: fluorescein diacetate (FDA)/ Sytox red, FDA/ Propidium iodide (PI), Syto 9/ PI, carboxyfluorescein diacetate (CFDA)/ Sytox red, Calcein AM/ Sytox red.

The fluorescent agents were dissolved in a 0.9% saline solution. The final concentrations adopted for the staining materials were FDA/ Sytox red: 25 µl ml^{-1} / 5 nmol ml^{-1}; FDA/ PI: 25 µl ml^{-1} / 2.5 µl ml^{-1}; Syto 9/ PI: 2 nmol ml^{-1}/ 20 nmol ml^{-1}; CFDA/ Sytox red: 25 µl ml^{-1} / 5 nmol ml^{-1}; Calcein AM/ Sytox red: 25 µl ml^{-1} / 5 nmol ml^{-1}; FDA/ ethidium bromide (Etbr):

25 µl ml^{-1} / 2.5 µl ml^{-1}; CFDA/ PI: 25 µl ml^{-1} / 2.5 µl ml^{-1}; TFDA/ PI: 25 µl ml^{-1} / 2.5 µl ml^{-1}; Syto 9/ Sytox red: 5 nmol ml^{-1} / 5 nmol ml^{-1}; Calcein AM/ PI: 25 µl ml^{-1} / 2.5 µl ml^{-1}.

After oral exposition for 120 min, specimens were transferred to the staining procedures immediately. Subsequently to the preparation with saline solution, the specimens were mounted with soft silicone (Gammasil Tec A 85 base) on the slide. The margins of the exposed enamel slabs were coated by the silicone impression material. A volume of 10 µl of the previously described fluorescence dyes was pipetted onto the prepared slabs on the slides and covered with the coverslips immediately. Micoscopic analysis followed directly afterwards. Only in the case of Syto 9/ PI (Baclight) the specimens were firstly incubated in the staining solution in a dark chamber for 10 min at room temperature. Afterwards, the slabs were mounted on the slide using superglue (Loctite 401, Loctite Deutschland GmbH, München, Germany).

After the staining procedures all specimens were analysed by epifluorescence microscopy. The analysis was conducted at 1000-fold magnification (Axioskop II, Zeiss; Oberkochen, Germany). The number of cells was counted in 10 randomized microscopic ocular grid fields (0,0156 cm^2 grid area). Two different light filters were used for discrimination of dead and viable cells (Etbr-filter for the visualisation of dead cells: BP 546/12; FT 580, LP 590; FDA-filter for vital cells: BP 450-490, FT 510 LP 515).

Colony forming units

After oral exposure the samples were removed from the splints and rinsed with 0.9% saline solution and transferred into sterile tubes with 1 ml 0.9% saline solution. Desorption of microorganisms was achieved by ultrasonication on ice for 1 min. The dispersed material was diluted up to 10^3 in saline solution and vortexed. A total of 100 µl of the diluted samples were plated out on Columbia blood agar (CBA, aerobic bacteria) and 100 µl on yeast-cysteine-blood agar, respectively (HCB, anaerobic bacteria). CBA plates were incubated for 2 days under aerobic conditions. HCB plates were placed in anaerobic candle jars at 37 °C using CO_2-Paks (BBL GasPak Anaerobic System Envelopes) for 5 d. After each incubation procedure the cultured colonies were counted. Low dilutions were determined using Quantity One 4.5 GelDoc EQ Universal Hood (BioRadLife Science Group, Hercules, USA). In order to obtain the number of bacteria per cm^2, the determined colonies of each plate were divided by the surface of the initial sample (0.196 cm^2).

Transmission electron microscopy

For each of the five different live/dead assays transmission electron microscopic analysis was performed. After oral exposure samples were incubated with the fluorogenic dyes. A control group was incubated after oral exposure but without the staining procedure in sterile NaCl-solution only. Rinsing with 1 ml phosphate buffer followed for all samples five times in succession for 10 min. Prior to analysis dehydration of the samples was performed using an ascending series of ethanol. Subsequently, the specimens were fixed in 4% paraformaldehyde / 0.1% glutaraldehyde for 2 h at 4°C. Before embedding in LR-White resin (London Resin company, Theale, Berks, UK) dehydration took place in an ascending series of ethanol. The enamel part of the embedded specimens was removed by decalcification using 1 M HCl. The remaining space was refilled with Araldite CY 212 (Serva, Heidelberg, Germany). A Mikrostar 45° diamond knife (Mikrotechnik, Bensheim, Germany) fixed in an Ultracut E microtome (Reichert, Heidelberg, Germany) was used to cut ultrathin sections in series. The ultrathin sections were mounted on 300 mesh nickel grids (Plano, Wetzlar, Germany).

The TEM-analysis was performed in an EM 902 microscope (Zeiss, Oberkochen, Germany) at an 80,000 fold magnification.

Statistics

Statistical evaluation was performed by ANOVA ($p \leq 0.05$) and the Tukey-test, enabling a pairwise comparison of the methods. In addition, the Kuskal-Wallis-test was carried out. All statistical evaluations were performed with the PASW Statistics 18 software.

Results

Live/Dead Staining

Basically, ten different staining techniques were evaluated (tab. 2), regarding different parameters such as the stability of staining, the differentiability of viable and dead bacteria, the general advantages and the disadvantages. Most of the staining combinations allowed a quantification of the cells immediately after the staining procedure but were characterized by a rapid fading of the fluorescence after 15 to 20 min. Only for Calcein AM/ Sytox red an incubation time for approximately 5 min after staining seemed to be necessary prior to quantification. Using the different filters for epifluorescence microscopic analysis, only with five assays clear differentiation of viable and avital bacteria was possible (FDA/ Sytox red, FDA/ PI, Syto 9/ PI, CFDA/ Sytox red, Calcein AM/ Sytox red). Detection of dead bacteria under both filters (FDA-filter and Etbr-filter) was achieved with the staining combination FDA/ PI (fig. 1). Analysis with the further combinations was inefficient due to lacking differentiability of viable and dead bacteria (CFDA/ PI, TFDA/ PI, Calcein AM/ PI and Syto 9/ Sytox red).

Based on these findings further investigation was performed using only five of the tested combinations (FDA/ Sytox red, FDA/ PI, Syto 9/ PI, CFDA/ Sytox red, Calcein AM/ Sytox red). Irrespective of the different stainings, the visualisation using the epifluorescence microscope showed randomly distributed adherent microorganisms on the enamel surface (fig. 1). Single cells, small aggregates and accumulation of cells could be visualized in an irregular manner. Vital and avital cells could be distinguished after staining, using two different microscope filters (Etbr-filter for the visualisation of dead cells: BP 546/12; FT 510, LP 515; FDA-filter for vital cells: BP 450-490, FT 510 LP 515). A differentiation between different bacterial species was not possible.

Quantification of all bacteria disregarding the different staining techniques and cell vitality yielded an average of $2.9 \pm 0.8 \times 10^4$ bacteria/cm². Viable cells and avital cells showed an equal distribution. Statistically, there were no significant differences between the number of viable bacteria (ANOVA: $p = 0{,}338$) and avital bacteria (ANOVA: $p = 0{,}413$). A maximum number of viable bacteria, 14.3×10^4 bacteria/cm², was ascertained using the staining combination FDA/ Sytox red (tab. 3). The same technique showed the minimum number of viable cells 0.8×10^4 bacteria/cm². The maximum of avital bacteria, 14×10^4 bacteria/cm², was detected with FDA/ PI. The minimum of avital cells, $0{,}5 \times 10^4$/cm² bacteria, was ascertained with Syto 9/ PI (tab. 3).

The five different live/dead assays for staining resulted in a wide range regarding the ratio of viable and avital cells (tab. 4). Based on the results, the assays were divided into three groups: quantification with Syto 9/ PI and Calcein AM/ Sytox red yielded an almost equal distribution of viable and avital bacteria. In the CFDA/ Sytox red and FDA/ Sytox red assays the monitored live/dead distribution was approximately 3:2. An aberrant dispersion was detected with FDA/ PI only (tab. 4). However, the differences between the techniques were not statistically significant (vital Tukey-test: p = 0,335; avital Tukey-test: 0,295). The additional Kruskal-Wallis-test showed no significant differences between the methods either (vital p = 0,530; avital p = 0,712).

Colony forming units

CFU showed a high interindividual and intraindividual variability. Comparing the mean number of aerobic and anaerobic bacteria, both groups are represented equally (aerobic: 89 ± 138.4 x 10^4 cm^{-2}; anaerobic: 99.1 ± 154.3 x 10^4 cm^{-2}). A correlation between the number of bacteria detected with CFU and the number of viable bacteria, detected with the staining techniques, could not be observed. The detected bacteria differed by 30 (total of detected viable bacteria using staining techniques: 2.9 ± 0.8 x 10^4 cm^{-2}).

Transmission electron microscopy

The electron-microscopic micrographs of specimens fixed after adoption of the different stains showed lysis and destruction of the adherent bacteria. Several types of destruction were visible as well as accumulation of the stains; no intact bacteria were found. However, TEM-micrographs of the unstained controls fixed in the native state showed intact bacteria. Accordingly, the different viability dyes lead to a destruction of the bacteria or the bacterial cell walls and cell membranes, respectively (fig. 2).

Discussion

Biofilms, consisting of extracellular polymeric substances (EPS) and bacteria, initialize oral infections such as caries and periodontopathy. However, little is known about the live/dead distribution during the initial process of bioadhesion in the oral cavity on dental hard tissues. Serving as a general pilot study, different live/dead assays were investigated. Smoothed bovine enamel specimens were used like in previous studies on bioadhesion and pellicle formation, an *in situ* approach based on upper jaw splints was chosen [1,32, 33]. Many studies are based on *in vitro* models to focus on special topics of biofilm formation, but bioadhesion *in vivo* and *in vitro* differ considerably [32]. *In situ* studies seem to be ideal if investigating physiological biodhesion with elaborate methods. Furthermore, bacteria in biofilms show twice to 1000 times higher resistance than planktonic bacteria indicating the necessity of studies on the viability of adherent bacteria in the oral environment [34].

Applying modern live/dead techniques, viable and dead bacteria were differentiated by their viability. But even though live/dead assays divide bacterial viability stages into dead and viable, there is still no standardized definition for a classification of the different bacterial states of viability [12]. Regarding the classical cultivation of microorganisms on agar plates, for example, only culturable bacteria, able to form colonies, are detected. Several bacterial non-sporulating species decrease under starvation conditions [35]. Furthermore, it has to be kept in mind that only 50 % of the bacterial strains present in the oral cavity are culturable [8]. Colwell et al. [36] describe these cells as ‘viable but non-culturable cells’ (VBNC), characterized by metabolic activity but loss of growth. The bacteria seem to pause in a state of ‘dormancy’, representing a reversible intermediate state as a kind of survival strategy, which can lead to active metabolism, death or lysis [30, 31]. Considering the fluorescence two-colour assays, the staining capacity of the dyes seems to correlate with the physiological state of the bacteria [37]: bacteria with active metabolism and high growth rate fluoresce more than dormant cells. Dormant cells may exhibit higher resistance und less fluorescing capacities because of a hindered cell penetration [38, 39]. Overall, 2 main principles for live/dead staining are presently used: impermeability of the membrane to nucleic acid dyes on one hand and presence of metabolic activity, on the other. It is questionable how cells in a stationary and therewith metabolic less active state interact in-depth with those reagents, based on the metabolic activity. Future studies are necessary to evaluate the principal interaction of the dyes with the bacteria, which was not the objective of the present study.

In this study, CFU were determined to validate the number of viable bacteria detected with the live/dead assays. Using CFU and the fluorescence based assays, it was shown, that a comparison of these methods seems not reasonable. Generated CFU data differed considerably from the number of stained viable cells. These findings were described in other studies before [1]. The desorption of adherent bacteria clusters from the enamel surface by ultrasonication could lead to a separation of aggregated cells and thus to a higher number of colonies. Also, the difference between the cultivability determined by CFU-method and the viability defined by fluorescence staining assays should be kept in mind. For further studies of the single stains, quantification of cells could be verified separately to the live/dead assays by other fluorescence-based methods, such as DAPI or FISH. The different techniques provide different information as vitality and bacterial identification of species. Furthermore, spatial analysis of biofilms could be analysed by confocal microscopy.

Even though most of the fluorescence based dyes detect viable cells by taking advantage of their metabolism, it was postulated that stained cells lose their viability shortly after intercalation of the dyes [24]. This hypothesis was confirmed by the TEM-analysis in the current study (fig. 2). The images showed immense lysis and destruction of the adherent cells as well as clustered stain throughout the surface induced by the different staining dyes indicating considerable aggressiveness of the adopted reagents. In contrast, the electron-microscopic micrographs of unstained controls showed intact adherent bacteria. This observation has to be kept in mind when interpreting the results of live/dead staining. All stained cells appear destroyed in equal manner regardless of their prior state of viability. Still, it seems quite plausible that only viable bacteria can accumulate dyes, which depend on membrane permeability or metabolic activity, indicating former viability of the respective bacterial cell. It can therefore be assumed, that the differentiation of viable and dead bacteria is possible, using different live/dead stainings. The analysis represents the viability state during the staining procedure. After dye-accumulation inside the cells, the bacteria indeed lose their viability. To quote an example, fluorescein diacetate only emits green light after intracellular metabolism and accumulation in the cell. On the other hand dead cell stains depend on damaged membranes for cell penetration. Staining with Propidium iodide, for example, requires loss of an intact membrane due to damage and thus cell death [40]. Accordingly, the effects observed with TEM offer a plausible explanation for the fading of the stained vital cells and the spreading of the dyes indicating dead bacteria, a phenomenon observed with several staining protocols in a different extent and velocity. Examining

ethidium bromide stained samples under the fluorescence microscope, for example, a rapid boost of deceased cells can be noticed. This dye particularly requires fast analysis.

Regarding the chosen fluorescing dyes and microscopic filter, respectively, only two different filters were used for all stains to visualize the viable and dead bacteria. Due to similar excitation and emission data of the different dyes, other optimised filters could have improved the visualisation after staining- but would most likely not influence the results. It can be assumed, that other filter would only result in a deeper intensity of the fluorescence. The used FDA-microscopic filter had a convenient side effect of also visualizing some of the fluorescence dyes for the avital bacteria, for example Propidium iodide. This effect could be used as an additional control for the differentiation of viable and dead cells, too.
Even though autofluorescence of the enamel surface existed, bacteria could be detected clearly.
A number of five assays were considered in the main experiments yielding different shares of viable and dead bacteria. Still, a certain variance between the different combinations of dyes seems characteristically. Altogether, distribution of viable vs. dead bacteria of the fluorescence assays resulted in three groups: equal distribution (Baclight and Calcein AM/ Sytox red); more dead bacteria vs. viable (FDA/ PI); more viable vs. dead bacteria (CFDA/ Sytox red and FDA/ Sytox red). The almost equal distribution of viable and avital bacteria was found, using BacLight (Syto 9/ PI) and Calcein AM/ Sytox red. While Calcein AM/ Sytox red staining are based on metabolic activity for the viable bacteria and impermeability for the avital cells, BacLight staining is characterized by the same staining principle of its combined dyes: both are based on membrane permeability. Syto 9 penetrates all viable and avital cells, PI only penetrates those cells with higher permeability of cell membrane. A remarkably high amount of dead bacteria was recorded with FDA/ PI indicating destructive properties of this combination. Also with the other dyes, 40 to 50 % dead bacteria were found. There are two possible explanations for this phenomenon. On the one hand dead bacteria might adhere, on the other hand lysozyme immobilised in the pellicle layer in an active conformation can destroy the cell membrane of some bacterial species. In general, combinations of dyes not overestimating the dead bacteria are preferable [1, 33]. Even though the high variability of the live/ dead distribution and of the CFU data points out the challenges of *in situ* biofilm models. The data gained with the different fluorescence-based assays, indicated distinct differences between each single staining combination as much as between each subject.

One possibility to overcome these inconstancies could be the application of an *in vitro* biofilm model, instead of the *in situ* model, used in the present study. Advantages of *in vitro* biofilm models are described in several previous studies, implying predictable reproducibility to a certain extent and specific composition of bacteria species [9, 40, 41]. However, defining the bacterial composition of the plaque model also leads away from the natural bioadhesion occurring *in situ/ in vivo* and thus might cause different biofilm characteristics. Also, repeatability of *in vitro* biofilm formations was investigated after more than 40 hours, while unity of the initial bacterial adherence remains still a challenge.
Anyhow, recent studies indicate that there is a subpopulation of cells that can take up PI during and immediately following exposure to stress but that a short incubation allows repair of the membrane damage. Davey and Hexley [42] showed that irrespective of the stress applied, approximately 7% of cells (*Saccharomyces cerevisiae*) exhibited the ability to repair. These results also underline the smooth transition between different viability states of bacteria.

All in all some of the tested live/ dead stainings are applicable for evaluation of the initial oral biofilm. Thereby the special characteristics of the respective assay are to be considered, adoption of other methods such as TEM are recommended in addition. Furthermore, the present data indicated clearly that viability assays performed with bacteria in the adherent state and the CFU-method after desorption yield completely different results and cannot be compared. Both offer different information.

Conclusions

The present study demonstrates adequate fluorescence-based techniques for the visualization and quantification of viable and dead cells, particularly of the initial bacterial adherence on *in situ* exposed enamel slabs (BacLight, FDA/ Sytox red, Calcein AM/ Sytox red and CFDA/ Sytox red). The methods tested are applicable alternatives to toxic and instable conventional assays. However, the disruptive effects of the dyes on the integrity of the bacterial membranes are to be considered.

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Conflict of interest The authors declare that they have no conflict of interest.

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Table 1 Fluorogenic dyes

Material	Manufacturer	Viability
*Bac*Light™ (Syto 9/PI, Bacterial Viability kit L7007)	Invitrogen Ltd., Paisley, UK	V, D
Calcein AM	Sigma-Aldrich Chemie GmbH, Steinheim, Germany	V
5(6)-carboxyfluorescein diacetate	Sigma-Aldrich Chemie GmbH, Steinheim, Germany	V
Etbr (1 %)	Carl Roth GmbH & Co., Karlsruhe, Germany	D
FDA	Sigma Chemical Co., St. Louis, USA	V
PI	Sigma-Aldrich Chemie GmbH, Steinheim, Germany	D
4,5,6,7-Tetrachlorofluorescein diacetate	Sigma-Aldrich Chemie GmbH, Steinheim, Germany	V
Sytox® red (dead cell stain)	Invitrogen Ltd., Paisley, UK	D

The chemicals were combined for the detection and visualization of dead (*D*) and viable (*V*) bacteria

Table 2 Synoptic overview of the different staining techniques

	Stability of staining	Differentiability of vital and dead bacteria	Advantages	Disadvantages	General rating
Syto 9/PI (BacLight)	Fading (after 15–20 min)	Obvious	Simplified preparation	10 min of incubation	+[a]
FDA/Etbr	High intensity, rapid fading	Obvious		High toxicity and fading	–[b]
FDA/PI	Adequate stability	Obvious	Viable and avital cells under FDA-filter		–[b]
CFDA/PI	Rapid fading	Difficult		Faintly fluorescence of avital bacteria	–[b]
TFDA/PI	Long-term stability	Vague		Stained background, difficult counting	–[b]
Calcein AM/PI	Rapid fading	Difficult			0[c]
Syto 9/Sytox red	Insignificant	Not countable		Stained background under FDA filter	–[b]
CFDA/Sytox red	Adequate visualization	Obvious			+[a]
FDA/Sytox red	Rapid fading of green fluorescence	Obvious			+[a]
Calcein AM/ Sytox red	Stable after 5 min of incubation	Not before incubation for 5 min			0[c]

[a] Reliable

[b] Inappropriate

[c] Adoptable

Table 3 Number of detected bacteria (10^4/cm²) with maximal and minimal data after incubation with the different techniques

The data refer to the viable and dead bacteria. Exposure time, t= 120 min; n=12 samples/technique, six subjects

Fluorogenic dyes	Viable	Dead	Viable		Dead	
	Mean ± SD	Mean ± SD	Min	Max	Min	Max
FDA/PI	2.2±1.1	4.2±3.9	0.8	4.7	1.1	14
FDA/Sytox red	4.3±4.5	3.2±3.2	0.8	14.3	0.6	10
Syto 9/PI	2.4±1.4	2.9±2.9	1	5.1	0.5	11
CFDA/Sytox red	2.5±1.4	1.9±1.0	1.1	4.7	0.8	3.9
Calcein AM/Sytox red	3.0±3.4	2.8±2.5	0.8	11.2	0.7	8.3

Table 4 Percentage distribution of viable and avital bacteria after adoption of the different staining techniques

Fluorogenic dyes	Viable	Avital
FDA/PI	34	66
FDA/Sytox red	58	42
Syto 9/PI	45	55
CFDA/Sytox red	57	43
Calcein AM/Sytox red	52	48

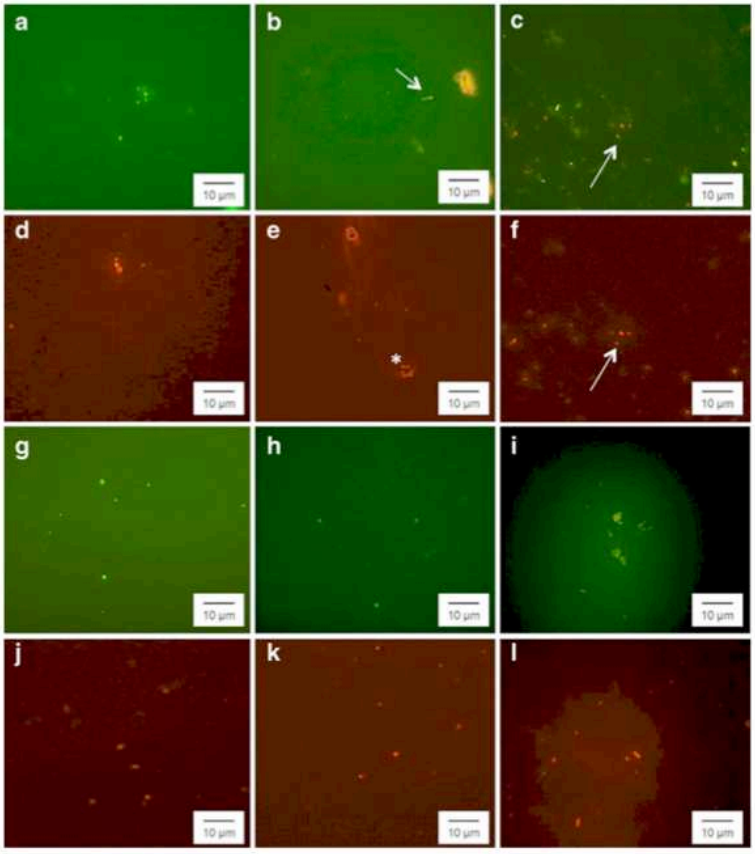

Fig. 1 Fluorescence microscopic visualization of adherent bacteria after adoption of the different live/dead stainings: FDA/Sytox red (**a**, **d**), FDA/PI (**b**, **e**), BacLight (**c**, **f**), Calcein AM/Sytox red (**g**, **j**), CFDA/Sytox red (**h**, **k**), and FDA/Etbr (**j**, **l**). Please note that single cells and aggregates of viable cells could be detected with the FDA staining (**a**), and single avital cells could be visualized with Sytox red (**d**). With the FDA/PI staining, avital cells could also be detected using the FDA filter (**b**, *arrow*) as well as the Etbr filter (**e**). Please note the avital and viable cells of the same section after BacLight staining using the FDA filter (**c**, *arrow*) and the Etbr filter (**f**, *arrow*). The Calcein AM/Sytox red staining shows a similar strong fluorescence of viable cells (**g**) and low-fluorescing avital cells (**j**). Randomly distributed viable CFDA- (**h**) and avital Sytox red-stained cells (**k**) could be visualized on enamel samples after oral exposure (t=120 min) using the FDA and Etbr filters. The staining combination FDA/Etbr demonstrated randomly distributed low-fluorescing viable (**i**) and avital cells (**l**) on enamel. Epifluorescence microscopy, original 1,000-fold magnification

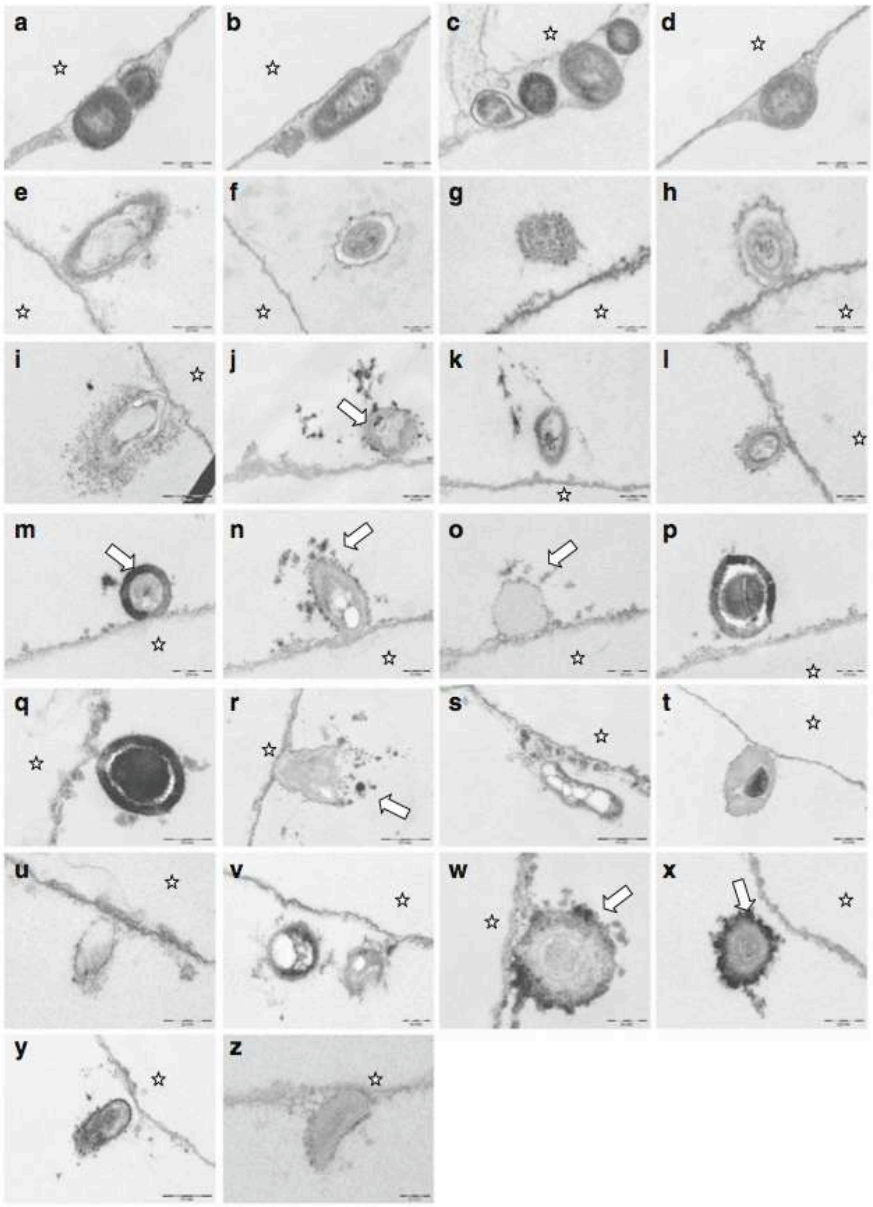

Fig. 2 TEM images of adherent bacteria on the in situ pellicle after staining with the different live/dead stainings. Different states of lysis and cell destruction are visible as well as accumulation of stains (*arrows*); no intact bacteria were detectable. Controls show typical bacteria with intact cell membrane. **a**–**d** Controls without application of fluorescent dyes, **e**–**h** BacLight (propidium iodide/Syto 9), **i**–**l** Calcein AM/Sytox red, **m**–**p** CFDA/Sytox red, **q**–**w** FDA/Sytox red, **x**–**z** FDA/propidium iodide. Please note that the enamel was removed during the embedding procedure by acid etching; the former enamel side is marked with an *asterisk*